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Hematophagous insects as vectors for frog trypanosomes

by

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Abstract: Experimental infections of three hematophagous arthropods *(Rhodnius prolixus, Aedes aegypti,* and *Culex pipiens)* with a trypanosome of the *Trypanosoma rotatorium* complex found in the frogs *Hyla crepitans* and *Leptodactylus insularum* revealed that *A. aegypti* is a good host for the flagellate; the course of development in the intestinal tract of the mosquito is described from 15 minutes to 168 hours. *C. pipiens* showed only low intestinal infections and *R. prolixus* did not permit development of the parasite.

It is postulated that, in addition to the transmission of *T. rotatorium* by leeches, batrachophilic mosquitoes may transmit the parasite to frogs of more terrestrial habits by being ingested by these anurans.

Most studies of the trypanosomes of poikilothermic vertebrates have been morphological. The relatively few investigations of the mode of transmission have incriminated various species of Psychodidae and Muscidae as vectors of reptilian trypanosomes (**Shortt** & **Swaminath,** 1931; **Ayala** & **McKay,** 1971) and leeches (Hirudinea) as vectors of frog trypanosomes (**Brumpt,** 1906). There is, however, evidence that certain hematophagous dipterans may serve as vectors of the batrachian trypanosomes. **Bailey** (1962) reported *Aedes aegypti* as an experimental host of *Trypanosoma rotatorium* from *Rana pipiens;* **Ayala** (1971) incriminated *Phlebotomus vexator occidentis* as a vector of the trypanosomes found in *Bufo boreas.* **Perez-Reyes** (1968) was unable to transmit *T. galba* by means of leeches, although he observed development of the parasite through various stages in *Culex quinquefasciatus* fed upon various species of infected Mexican frogs. **Desser** *et al.* (1973) observed the extrinsic life cycle of *T. rotatorium* from *R. clamitans* within *Culex territans,* also noting that leeches had never been observed parasitizing the adult frogs, and that tadpoles of the species, living in the same water with the leeches, were uniformly negative when examined for the parasite.

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The present work investigates the possibility of development of frog trypanosomes to the infective stage in certain bloodsucking insects, a triatomid and two culicines known to be attracted to, and to feed upon, batrachians (**Henderson & Senior,** 1961; **Urdaneta-Morales & McLure,** 1972; **Woke,** 1937).

MATERIAL AND METHODS

All insects used were laboratory-reared. Second-stage nymphs of *Rhodnius prolixus* were derived from our laboratory strain, maintained at 29 C and 70-80% relative humidity, following the methods of **Gomez-Núñez & Fernández** (1963). The "Caracas" strains of *Aedes aegypti* and *Culex pipiens* were long-maintained laboratory strains, while the "El Veladero" strains of both species were derived from eggs laid by wild-caught females from the same site where most of the frogs were captured. They were bred according to the techniques of **Scorza** (1972) and kept in the laboratory at 26 $\pm$ 1 C.

The frogs studied were *Colostethus trinitatis* (Dendrobatidae) from "Quebrada de Chacao", very near Caracas; *Bufo marinus* (Bufonidae) from Caraballeda on the seacoast north of the city; *Hyla crepitans* (Hylidae); *Leptodactylus insularum, L. macrosternum, L. sibilatrix* (Leptodactylidae); and *Pseudis paradoxus* (Pseudidae). The latter five species were all collected in "El Veladero" in the plains of the State of Guárico, about 100 km southwest of Caracas. The frogs were maintained in the laboratory by the techniques of **Scorza** and **Dagert** (1958).

Each frog, on arrival at the laboratory, was examined thoroughly for adherent leeches on the skin. In addition, blood was examined for trypanosomes, either fresh under 400 X phase contrast, or in films fixed 3 minutes in methanol and stained 45 minutes in 5% Giemsa in a phosphate buffer (pH 7.2). Large frogs were bled by cutting one digit, while smaller frogs were bled by hungry first-stage nymphs of *R. prolixus*, an adaptation of the method of **Scorza** (1971) for bleeding lizards. Frogs which did not show natural parasitemias were used in the experimental infections.

Experimental infection of insects: Twenty second-stage *R. prolixus*, 30 adult *A. aegypti*, and 30 adult *C. pipiens* were examined by the techniques of **Garnham** (1966) and **Guedes** (1952) to detect the possibility of their harboring natural infections of Trypanosomatidae similar to those reported from triatomids (**Cerisola *et al.*,** 1971) and culicines (**Steinhaus,** 1947).

Seventy second-stage nymphs of *R. prolixus*, which had fasted for 15 days, were allowed to engorge upon heavily parasitized *H. crepitans*, and 150 specimens each of *A. aegypti* and *C. pipiens*, one week old and fasted for 48 hours, were fed on *H. crepitans* and *L. insularum* with elevated parasitemias. Once engorged, the insects were kept under the above mentioned conditions and the digestive tracts of representative specimens dissected at the following intervals: 15 min, 30 min, 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h. The intestinal material thus obtained was examined in fresh condition under phase contrast and in smears stained with Giemsa to search for developing stages of the parasites, which, when found, were photographed on Plus X Pan film under oil immersion at 1000 X. In addition, those lots of insects to be sacrificed at 144 and 168 h were previously placed in 6 x 4 cm glass tubes with 1-2 drops of sterile saline in the bottom, to collect any feces which might contain developing parasites.

Experimental infection of batrachians: Once the infectivity of the trypanosomes to the insects had been established, it was decided to investigate the possible mechanism of infection of the vertebrate hosts. To this end, the intestinal contents of 80 *A. aegypti* which contained large numbers of intestinal flagellates 48 h after being fed on infected *L. insularum* were pooled and mixed with sterile saline. Aliquots of this inoculum were injected intraperitoneally into 1 *H. crepitans*, 1 *L. insularum*, 1 *P. paradoxus*, and 2 *B. marinus*. Concurrently, 2 specimens of each of these species were injected subcutaneously with the inoculum. At the same time, 5 infected mosquitoes were force-fed to 2 specimens of each species of frog, and 15 infected mosquitoes fed to each of 2 *B. marinus*. On the 4th day after the experimental infections, and at 4 day intervals thereafter for 2 months, blood from each animal was examined under phase contrast, the number of parasites per 50 fields at 400 X being counted.

RESULTS

With the exception of *B. marinus*, all species of anurans showed parasitemias of a trypanosome which was accepted as belonging to the *rotatorium* complex (Fig. 1). The trypanosomes were very large, foliaceous, and with the typical rotary movement of this species. Some of the flagellates were long and slender, and others were of intermediate form; this polymorphism is taken to be characteristic of the species (**Scorza** & **Dagert**, 1958). Leeches were not found on any of the frogs.

The intestinal contents of *R. prolixus*, *A. aegypti*, and *C. pipiens* examined under phase contrast to detect possible natural infection with insect flagellates proved uniformly negative.

Experimental infections of insects: When the intestinal contents of specimens taken from the 70 nymphs of *R. prolixus* fed upon highly parasitized *H. crepitans* were examined at 15 min, 30 min, and 60 min after engorgement, essentially normal blood trypanosomes were seen, accompanied by others which were rounded, of slow or no movement, and whose cytoplasm was clear and fragmented; these signs of degeneration increased until, between 48 and 168 h, only amorphous, hyaline masses of cytoplasm were observed. It was thus concluded that *R. prolixus*, as previously reported by **Pessoa** (1969), does not permit the development of *T. rotatorium* in its digestive tract.

In 150 specimens of the "Caracas" strain of *A. aegypti* fed upon highly parasitized *H. crepitans* and *L. insularum*, the sequence of events was as follows:

1. Phase contrast examination of the intestinal content of mosquitoes dissected from 15 min to 6 h after feeding showed a certain number of apparently normal blood flagellates, together with others that moved slowly, were apparently losing the undulating membrane, and were becoming oval or rounded. These latter forms appeared in the stained material as unflagellated large bodies with a pink-stained nucleus having an intensely purple-stained kinetoplast superimposed. Finally, these parasites became completely rounded, cyst-like bodies which would correspond to those observed by **Desser** *et al.* (1973) in *C. territans* 1 h after feeding on *R. clamitans* (Fig. 2).

2. From 6 to 12 h, the blood forms became completely modified and were seen in very low numbers. There were present many "pseudocysts"

which fragmented with the formation of numerous "rosettes" composed of 4-30 flagellates of the sphaeromastigote type (Fig. 3). Organisms similar to epimastigotes were seen, though in very low numbers, in the 12 h material.

3. In the 24 h material stained with Giemsa the blood forms were transformed into "rosettes". One type was composed of small sphaeromastigotes, while another was composed of large numbers of epimastigote-like forms (Fig. 4). There were two types of epimastigotes, one broad and short and the other long and slender.

4. Forty-eight hours after feeding, the blood content of the digestive tract of the insects had decreased to the point that it was possible to separate the stomach from the intestine and examine these organs separately. In the stomach there were sphaeromastigotes and very large numbers of epimastigotes, many in the process of binary division, especially the long, slender type. In the hind gut, flagellates (epimastigote type) appeared for the first time.

5. Seventy-two hours after feeding, the majority of mosquitoes showed only traces of blood in the digestive tract, and correspondingly few flagellates (epimastigotes) in the stomach. Some mosquitoes had large numbers of this form in the stomach; the hind gut showed traces of blood also, together with freely moving epimastigotes; 2 insects had large numbers of this form in the hind gut. Some of the parasites still occurred in "rosettes".

6. Ninety-six hours after feeding, the majority of mosquitoes showed neither blood residue nor parasites in the whole length of the digestive tract, although a few slowly moving epimastigotes were seen in the hind gut of some insects.

7. From 96 to 168 h, no mosquito dissected had any parasites in any part of the digestive tract.

The fecal material expelled by infected *Aedes* contained a few slow-moving and apparently moribund flagellates of the epimastigote type; sphaeromastigotes were not observed.

No insect at any time had parasites in the salivary glands or the Malpighian tubules.

The "El Veladero" strain of *A. aegypti* gave results similar to the "Caracas" strain.

The "Caracas" and "El Veladero" strains of C. *pipiens* were very reluctant to feed on frogs by day or by night. During the day, although covered by a black cloth and given an opportunity to feed for 4 hours, or during the night (11 P.M. - 2 A.M.) the majority refused to feed, and there was never complete engorgement; **Jordan** (1961) reported similar behavior for this species feeding on *R. pipiens*.

Sixty *C. pipiens* placed with *H. crepitans* at night were negative for flagellates in every examination under phase contrast; on the other hand, of 90 *Culex* fed by day on *L. insularum* with high parasitemia, a few showed low numbers of sphaeromastigotes and epimastigotes, some in "rosettes", 24 h after feeding. The greatest numbers of intestinal flagellates were observed between 48 and 72 h, but always in far lesser quantities than in *Aedes*. After 72 h, the number of flagellates in *C. pipiens* was insignificant.

In attempts at experimental infection of batrachians, neither *B. marinus* nor *P. paradoxus* developed infections. The highest parasitemia (18 parasites/50 fields at 400 X) was obtained in an orally infected *H. crepitans*, 27 days post-infection. *L. insularum* showed parasitemias of 10 parasites/50 fields after any of the three modes of infection.

DISCUSSION

Southworth *et al.* (1968) showed that *T. rotatorium* had a diurnal periodicity in batrachians, during daylight hours the parasites were found in the peripheral circulation, and at night, most of them retreated to the viscera. **Bardsley** and **Harmsen** (1969), in their experiments on *R. catesbeiana*, found that the trypanosome is directly influenced by temperature and by the activity of the host: the parasites tending to migrate to the peripheral circulation when temperature and activity are high. This led them to suspect that the vector of *T. rotatorium* might not be aquatic, since the basking habit of many species of frogs would remove them from the water at the time when the peripheral parasitemia would be highest, so that the parasites would be more available to hematophagous dipterans than to leeches.

In our experiments, of the three methods of infection employed for the batrachians, the best results were from oral infection of *H. crepitans;* of the few prior attempts to infect frogs or toads, the best results have come from feeding infected Psychodidae to *B. boreas* **(Ayala,** 1971).

To date, no experiments, including these, have demonstrated the development of frog trypanosomes in insects to the metatrypanosome stage. Our experimental infections were carried out with material in which no metatrypomastigotes were seen, either in fresh or stained material. *T. rotatorium* has not been seen to develop to the metacyclic stage in either *A. aegypti* or in *C. territans* **(Bailey,** 1962; **Desser** *et al.*, 1973). **Perez-Reyes** (1968) managed to infect *R. palmipes* and *R. montezumae* with cultures of *T. galba* in which no metacyclic forms were visible.

These considerations suggest the hypothesis that transmission of batrachian trypanosomes may occur in two different ways: by leeches in those anurans of more strictly aquatic habits, and by dipterans in the frogs and toads which spend a relatively greater part of their time on land. In the former mode of transmission, the leeches infect the batrachians with the infective parasites that are present in their mouth parts (infection from the anterior station). In the latter, the dipterans (Psychodidae and Culicidae) which are attracted to and feed upon toads of almost exclusively terrestrial habits *(Bufo boreas, B. marinus)* or frogs in which the adults are absent from the water for long periods *(Rana catesbeiana, Hyla crepitans, Leptodactylus insularum)* would infect the vertebrate hosts by being ingested. It would appear that, in this case, the infective stage would be the epimastigote rather than the metatrypomastigote, as also occurs in the trypanosomiasis of lizards **(Ayala,** 1973; **Ayala & McKay,** 1971).

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RESUMEN

De los resultados logrados al intentar infectar experimentalmente tres especies de artrópodos hematófagos *(Rhodnius prolixus, Aedes aegypti* y *Culex pipiens)* con un tripanosoma perteneciente al complejo *Trypanosoma rotatorium,* albergado por *Hyla crepitans* y *Leptodactylus insularum* se deduce que *A. aegypti* es un buen huésped para este parásito; *C. pipiens* mostró infecciones intestinales muy bajas, en tanto que *R. prolixus* no permitió el desarrollo del flagelado.

Se detalla el ciclo extrínsico del parásito que se va desarrollando en el tracto intestinal de *A. aegypti* desde 15 minutos hasta 168 horas después de la comida sanguínea infectante.

Se plantea, a manera de hipótesis, la posibilidad de que las tripanosomiasis de batracios puedan transmitirse no sólo a través de hematófagos acuáticos (Hirudíneos) sino también, en algunas especies de anuros, mediante la ingestión de dípteros hematófagos batracofílicos *(A. aegypti).*

LITERATURE CITED

Ayala, S. C.
1971. Trypanosomes in wild California sandflies and extrinsic stages of *Trypanosoma bufophlebotomi. J. Protozool.,* 18: 433-436.

Ayala, S. C.
1973. The phlebotomine sandfly-protozooan parasite community of Central California grasslands. *Amer. Mid. Nat.,* 89: 266-280.

Ayala, S. C., & J. G. McKay
1971. *Trypanosoma gerrhonoti* n. sp. and extrinsic development of lizard trypanosomes in California sandflies. *J. Protozool.,* 18: 430-433.

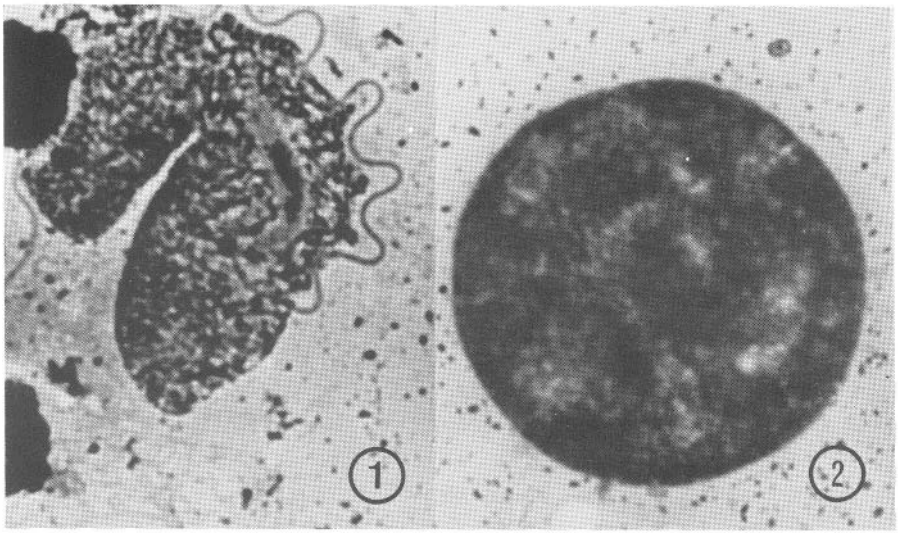
Fig. 1. Blood trypomastigote of *Trypanosoma rotatorium* from *Hyla crepitans.* Giemsa stain, 1000 X.

Figs. 2-4. Developing stages of *T. rotatorium* taken from the digestive tracts of *Aedes aegypti,* dissected at varying intervals after feeding on *Hyla crepitans.* Giemsa stain, 1000 X.

Fig. 2. "Pseudocyst", 30 min after blood ingestion.

Fig. 3. Fragmentation of "pseudocyst", 12 hs after blood ingestion.

Fig. 4. "Rosette" of parasites, 24 hs after blood ingestion.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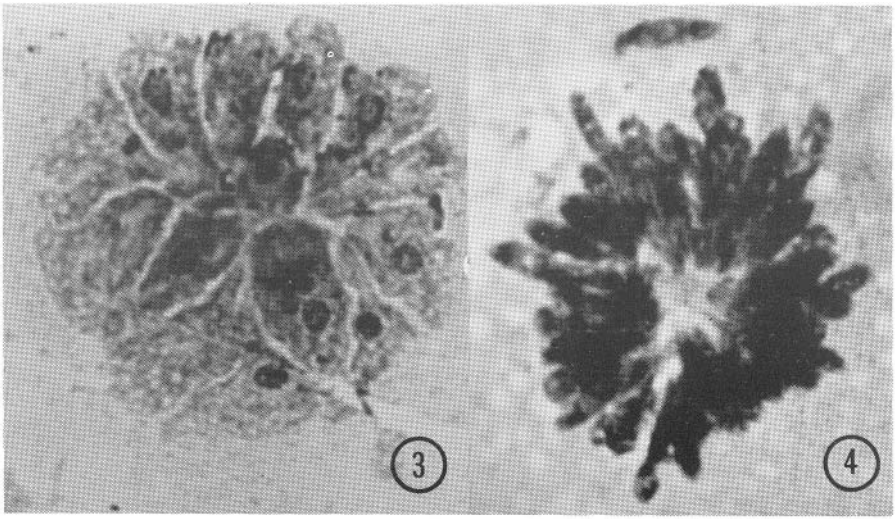
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Bailey, J. K.
1962. *Aedes aegypti* as a possible new invertebrate host for frog trypanosomes. *Exper. Parasit.*, 12: 155-163.

Bardsley, J. E., & R. Harmsen
1969. The trypanosomes of Ranidae. I. The effects of temperature and diurnal periodicity on the peripheral parasitemia in the bullfrog *(Rana catesbeiana* Shaw). *Can. J. Zool.*, 47: 283-288.

Brumpt, M. E.
1906. Role pathogéne et mode de transmission du *Trypanosoma inopinatum* Ed. et Et. Sergent. Mode d'inoculation d'austres trypanosomes. *Compt. Rend. Soc. Biol. Paris,* 61: 167-169.

Cerisola, J. A., C. E. del Prado, R. Rohwedder, & J. P. Bozzini
1971. *Blastocrithidia triatomae* n. sp. found in *Triatoma infestans* from Argentina. *J. Protozool.*, 18: 503-506.

Desser, S. S., S. B. McIver, & A. Ryckman
1973. *Culex territans* as a potential vector of *Trypanosoma rotatorium.* I. Development of the flagellate in the mosquito. *J. Parasit.*, 59: 353-358.

Garnham, P. C. C.
1966. *Malaria Parasites and other Haemosporidia.* Blackwell Scient. Pub., Oxford, 1114 p.

Gómez-Núñez, J. C., & M. J. Fernández
1963. La colonia de *Rhodnius prolixus* en el Instituto Venezolano de Investigaciones Científicas. *Bol. Inform. Direc. Malariol. San. Amb.,* 3: 132-137.

Guedes, A. D.
1952. Determinação do indice de infecção de Triatomineos por *Schizotrypanum cruzi* pelo exáme simples de fezes obtidas por expressão e por disecção do intestino posterior do inseto. *Rev. Brasil. Malariol. Doenças Trop.,* 4: 433-436.

Henderson, B. E., & L. Senior
1961. Attack rates of *Culex tarsalis* on reptiles, amphibians and small mammals. *Mosquito News,* 21: 29-32.

Jordan, H. B.
1961. The effects of the quality of blood and temperature on the production and viability of eggs in *Culex quinquefasciatus. Mosquito News,* 21: 133-135.

Perez-Reyes, R.
1968. *Trypanosoma galba* n. sp., parásito de ranas mexicanas. Morfología y ciclo en el vertebrado. *Rev. Lat-amer. Microbiol. Parasit.*, 10: 79-84.

Pessoa, S. B.
1969. Experiencias sôbre a transmissão do *Trypanosoma cruzi* por sanguessugas e de tripanosomas de vertebtados de sangue frio por triatomineos. *Rev. Saude Publ., S. Paulo,* 3: 17-20.

Scorza, J. V.
1971. Some haematological observations on *Tropidurus torquatus* (Sauria, Iguanidae) from Venezuela. *J. Zool.*, 165: 557-561.

Scorza, J. V.
1972. *Observaciones bionómicas sobre* Culex pipiens fatigans *Wied., 1829 de Venezuela.* Edcs. del Rect. Univ. de los Andes. Mérida, Venezuela. 198 p.

Scorza, J. V., & C. Dagert
1958. Sobre la sinonimia del *Trypanosoma rotatorium* Mayer, 1843, en batracios de Venezuela. *Bol. Venez. Lab. Clin.,* 3: 29-36.

Shortt, H. E., & C. S. Swaminath
1931. Life-history and morphology of *Trypanosoma phlebotomi* (Mackie, 1914). *Ind. J. Med. Res.,* 29: 541-563.

Southworth, G. C., G. Mason, & I. R. Seed
1968. Studies on frog trypanosomiasis. I. A 24 hour cycle in the parasitemia level of *Trypanosoma rotatorium* in *Rana clamitans* from Louisiana. *J. Parasit.,* 54: 255-258.

Steinhaus, E. A.
1947. *Insect Microbiology.* Comstock Publ. Co., Inc., New York, 763 p.

Urdaneta-Morales, S., & I. McLure
1972. Observations upon Haematophagy in Venezuelan Triatomids fed upon Poikilotherms. *Acta Cient. Venezolana,* 23: 161-164.

Woke, P. A.
1937. Cold-blooded vertebrates as hosts for *Aedes aegypti* Linn. *J. Parasit.,* 23: 310-311.